Comparison of Wild-Type and Mutant *white eye* Alleles in Melon Fly (Diptera: Tephritidae)

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ABSTRACT The DNA sequence of the coding region of the wild-type and mutant alleles of the *white eye* gene from the melon fly, *Bactrocera cucurbitae* Coquillett, was obtained. The mutant *white eye* allele had a single base pair mutation at the 5′ end of intron 4b in the RNA splice recognition site. Due to the disrupted splice recognition site, intron 4b was not removed from the mutant RNA transcript. The resulting mRNA transcript was 68 bp longer than that of the wild type, containing a frameshift and premature stop codon. Transport of pigment precursors would be blocked, resulting in a lack of pigmentation deposition consistent with the known mutant phenotype.

KEY WORDS melon fly, white eye, sterile insect technique, Tephritidae, transmembrane protein

THE MELON FLY, *Bactrocera cucurbitae* Coquillett, was introduced into Hawaii in 1895 (Clausen et al. 1965). In addition to Hawaii, this species is established in Guam, New Guinea, Okinawa, Rota, Africa, and Southern/Southeastern Asia (White and Elson-Harris 1992). As its name implies, hosts of *B. cucurbitae* include many species in the family Cucurbitaceae (e.g., cucumber, pumpkin, watermelon, and squash; White and Elson-Harris 1992). Male annihilation and bait sprays are the two primary methods of control; however, both pose environmental and human health concerns due to the use of pesticides (Mitchell et al. 1995). The sterile insect technique (SIT) is a nontoxic alternative that uses multiple releases of irradiation-sterilized males, which then mate with wild females, rendering them sterile. This results in a reduction in the wild population over time. However, the presence of released sterile females in SIT effectively reduces the suppression efficacy by permitting released females to compete with wild females as mates for the sterile males (McInnis et al. 1996, Lance et al. 2000). Production of males for SIT is problematic because it is not possible to separate the sexes before the adult stage. Elimination of the females early in the production process would reduce costs by up to 50%. In addition, the efficiency and acceptability of SIT is realized by eliminating the sting damage to fruit caused by the released, sterile females when they attempt to oviposit (McInnis et al. 1996, Lance et al. 2000).

Current genetic research is directed primarily at developing genetic sex sorting systems to enhance the effectiveness of SIT (Robinson 1989). Melon fly sexing strains based on flightless mutations (McCombs et al. 1993) and pupal color dimorphism (D. O. McInnis, personal communication) require that both sexes be reared until separation occurs at the adult or pupal stage, respectively. We propose use of transgenesis for development of novel strains that provide sex sorting at an early stage of development. This would require a selectable marker gene, e.g., *white eye*, in combination with an efficient vector, e.g., *piggyBac*, as demonstrated in other tephritid fruit flies, *Ceratitis capitata* (Wiedemann) (Handler et al. 1998) and *Bactrocera dorsalis* (Hendel) (Handler and McCombs 2000). The *white eye* marker could be used to add a beneficial sexual dimorphism gene (based on genomic studies of *Drosophila melanogaster* Meigen) into a mutant strain.

The melon fly *white eye* mutant strain is a result of a spontaneous mutation found during rearing at the USDA Manoa facility. This strain was backcrossed to ensure that it was a single recessive gene. Eye color mutations have been observed in many species of fruit flies. There are two pathways of light-screening pigments in *D. melanogastor* (Tearle 1991). These are composed of the brown ommochromes and red pteridines. At least three genes, *white*, *scarlet*, and *brown*, function in transmembrane movement of pigment precursors in these pathways. It is believed that the *white* gene product forms a heterodimer with either the *scarlet* or *brown* gene product, and this transmembrane protein complex moves substrates into several types of organ cells and cellular compartments. The ommochrome pathway begins with transport of tryptophan into the fat body and Malpighian tubules of larvae. During this larval storage phase, the tryptophan in the fat body is converted to kynurenine. Tryptophan in the Malpighian tubules is converted to kynurenine and then to 3-hydroxykynurenine. In the

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adult, these precursors are moved into the compound eyes and ocelli, and further processed into pigments. Defective *white/scarlet* protein heterodimers in the plasma membrane blocks the movement of ommochrome precursors into and throughout the compound eye (Tearle 1991). Lack of brown-colored ommochromes leaves only the red-colored pteridine pigments, resulting in the *scarlet* mutant phenotype. The pteridine pathway is less understood; however, blockage of these red pigments results in the *brown* mutant phenotype. The *white* phenotype results in a blockage of both pathways, neither pteridine nor ommochrome pigments are produced, and the eye is devoid of color.

Experimental Procedures

Insect Rearing. The melon fly wild-type stock strain (*cue*) and mutant *white eye* strain (*we*) were maintained in the Department of Plant and Environmental Protection Sciences at the University of Hawaii at Manoa. The *cue* strain was obtained from the Manoa USDA–ARS (Honolulu) mass-rearing colony and was maintained in culture for >15 yr. The *we* mutation occurred as a spontaneous mutation in the wild-type colony maintained by the USDA–ARS (McCombs et al. 1996). The *we* mutant was isolated and maintained in culture for ≈5 yr before initiation of this study. Flies were reared on artificial diet (McCombs et al. 1993) and had an egg-to-egg generation time of ≈34 d under standard laboratory rearing conditions (24°C, 62% RH, 24-h light).

DNA Isolation. One-day-old pupae were used for DNA isolation. Initial nucleotide extraction followed protocols developed for the oriental fruit fly (Chang 1995, Xiao 1997). The Wizard genomic DNA purification kit (Promega, Madison, WI) was used during the second half of the research project. One- to 3-h-old pupae from both mutant and wild-type melon fly strains were used for DNA extraction according to the manufacturer's protocol for a mouse tail.

DNA Quantification. Agarose gel electrophoresis was used to estimate the quality and quantity of the isolated DNA. A 1.0% agarose gel was prepared with 40 mM Tris-acetate, 1 mM EDTA (TAE) or Tris borate-EDTA (TBE) (Sambrook and Russell 2001), stained with ethidium bromide, and run in 1× TAE (or 0.5× TBE) at 69 V. A 0.25 μg/ml λHINDIII (MBI Fermentas, Hanover, MD) sample was used as a molecular weight marker for size and concentration comparison. Samples also were analyzed by a spectrophotometer.

DNA Polymerase Chain Reaction (PCR). The major portion of the *white eye* gene was obtained from PCR of genomic DNA. Multiple primer combinations were used to span the coding region of the gene to derive a single consensus sequence (Fig. 1). The furthest downstream genomic primer was at position 2273 and upstream of the polyA addition site (Fig. 1).

Standard 50-μl reactions for PCR consisted of dNTPs (200 μM each; Promega), Thermo buffer B (1×; Promega), $Mg(OAc)_2$, or $MgCl_2$ (2.5 mM), *Taq* polymerase (1.25 U; Promega), two primers (400 pmol each), DNA template (≈100 ng), and water. The positive control was a plasmid, containing DNA coding for a conserved region of the *white eye* gene. The microtubes were placed in a heating block and run with the following basic program: 94°C for 4 min [30 cycles at 94°C for 30 s/58°C for 45 s/72°C for 90 s], and a final 10-min stage of 72°C. The primer sequences were derived from two sources. The initial primer sets were those used by Xiao (1997) to sequence the oriental fruit fly *white eye* gene. Additional primers were designed from the melon fly *we* sequence as it became available to improve the specificity of the PCR and to determine the entire gene sequence.

RNA Isolation. Third instars were collected for RNA isolation from both the wild-type and *we* melon fly strains. The anterior one-third of the larvae were removed with a scalpel and immediately frozen with liquid nitrogen. Messenger RNA (mRNA) was isolated from third instar heads and used to construct cDNA. The SV total RNA isolation system (Promega) was used to extract total RNA. Manufacturer protocols were followed for both kits. Total RNA and mRNA was quantified using a spectrophotometer.

3′ Rapid Amplification of Complimentary DNA Ends (RACE) and Reverse Transcription Polymerase Chain Reaction (RT-PCR). The 3′ tail of the *white eye* gene was amplified using a combination of nested primers, gel band excision and purification, and 3′ RACE (Invitrogen, Carlsbad, CA) protocol. Complimentary DNA was constructed using the 3′ RACE kit (Invitrogen) or PCR-select cDNA subtraction kit (BD Biosciences Clontech, Palo Alto, CA) with either mRNA or total RNA as a template. Full-length cDNA transcripts were reacted with primers identical to genomic PCR. Genomic sequences were confirmed and intron/exon positions were determined by RT-PCR.

Inverse PCR. The sequence 5′ of the start codon (positions −1182 to 0; Fig. 1) was determined by inverse-polymerase chain reaction (IN-PCR). This was accomplished by designing primers on the 5′ side of the *Bst*ZI cut site "CGGCCG," located at positions 56–61. Genomic DNA was digested with *Bst*ZI restriction enzymes (Promega) and then religated in a circular manner. Primers for inverse PCR were made from exon 1 cDNA data. Long PCR was performed with circularized DNA, gene specific primers, and eLONGase (Invitrogen) DNA polymerase.

Cleaned PCR products were cloned using the Invitrogen pCR2.1 vector system. The gene cleaned sample was integrated into the vector via manufacturers' protocols. Inverse PCR samples with positive gel bands were pelleted and purified for PCR and sequencing using the High Pure plasmid isolation kit (Roche Diagnostics, Indianapolis, IN) according to manufacturer's protocols. Samples were run on a gel to check for correct size bands. Invitrogen cloning primers were used to sequence the inverse PCR products.

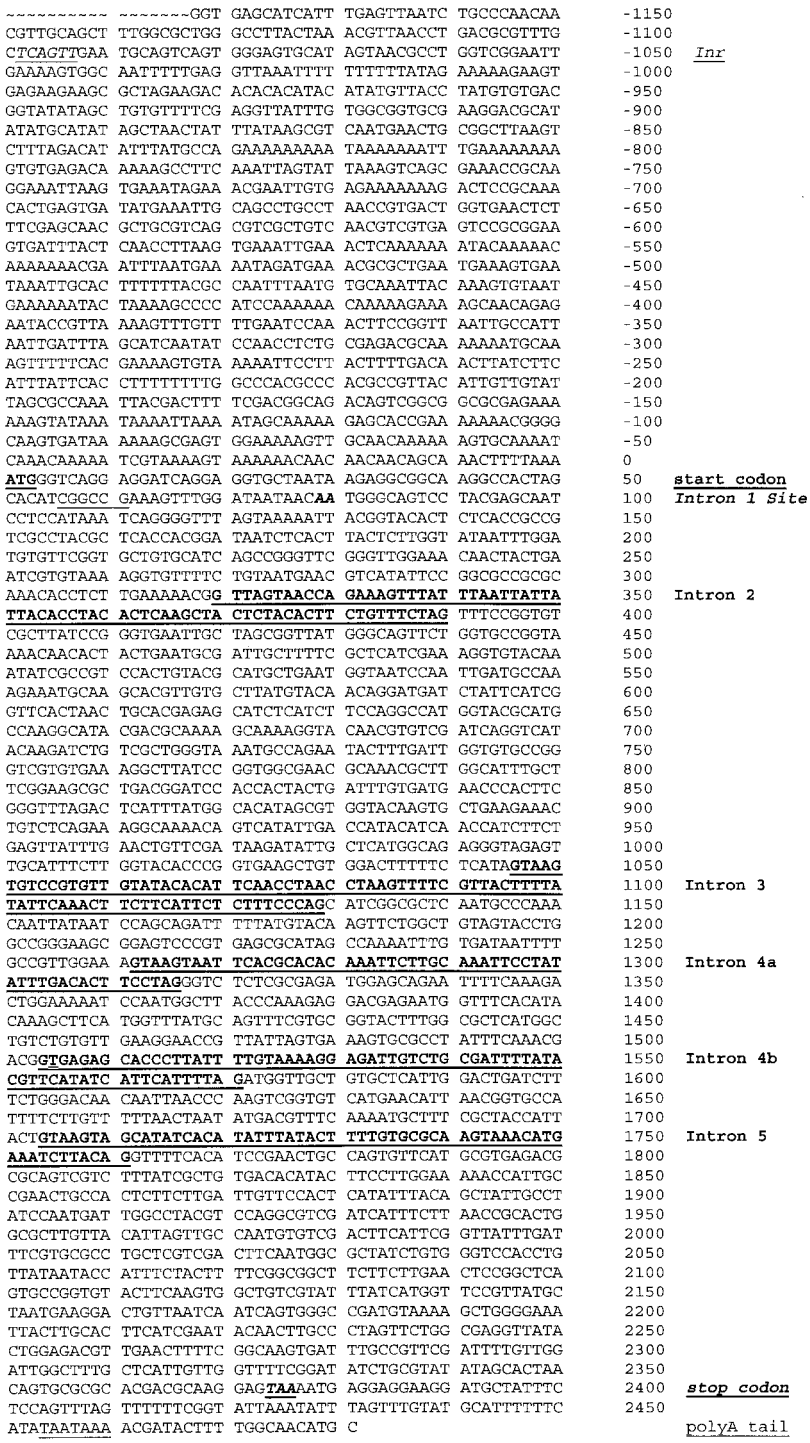

Fig. 1. Nucleotide sequence of the *B. cucurbitae* wild-type *white eye* coding region. Spans exons 1–6 and introns 2–5; intron 1 is not available but is located between nucleotides 79–80 (GenBank accession nos. AY155346 and AY155345). IN-PCR *Bst*ZI restriction enzyme cut site at nucleotides 56–61. Nucleotide 1505 is altered in the mutant allele, disrupting the intron 4b splice site.

Alignment of the cDNA and Genomic Sequences. PCR products having single bands were purified with the High Pure PCR product purification kit (Roche Diagnostics). PCR products with multiple bands had particular bands excised and purified with the Agarose gel DNA extraction kit (Roche Diagnostics) or Gene Clean (Bio 101, Vista, CA). PCR product and gel purification kits were used according to manufacturer's protocols. Samples were prepared and sent for automated sequencing to the Biotechnology/Molecular Biology Instrumentation and Training Facility, University of Hawaii at Manoa.

The genomic DNA and cDNA sequences were compared using the Genetics Computer Group program (version 10.1; Madison WI) to determine exon positions. The remaining sequences were either introns or control sequences that regulate gene expression. The mutant and wild-type *we* sequences were aligned to determine the basis of mutation in the *we* alleles.

Results and Discussion

***White eye* Phenotype.** The compound eyes of the wild-type melon fly have a deep red pigmentation and a blue-green iridescence (McCombs et al. 1996). The eyes of the *white eye* mutant strain are completely devoid of color. This is contrasted with other tephritid eye color phenotypes (e.g., *scarlet* and *brown*); these mutations have some form of pigmentation in the compound eye, however faint it may be. In *white eye* strains of other tephritids, mutations in the *white* gene are associated with changes in the pigmentation patterns of other tissues as well. For example, the Malpighian tubules of the *white eye* mutant of the oriental fruit fly are not pigmented, in contrast to the bright yellow wild-type tubules (Xiao 1997). The melon fly strains in this study were dissected to examine the internal organs for similar tissue-specific phenotypic differences. The Malpighian tubules of the wild-type mature third instar were bright yellow. The mutant Malpighian tubules do not have pigmentation. This may be due to lack of tryptophan transport and/or synthesis of 3-hydroxykynurenine.

Wild-Type Consensus Sequence. The wild-type consensus sequence (Fig. 1) and structure was determined. Intron 1 is typically a very large portion of the *white eye* gene and was not sequenced in this study. The first intron of *Bactrocera tryoni* (Froggatt), for example, was determined to be 12 kb in length (Bennett and Frommer 1997). The melon fly wild-type *white eye* sequence, excluding intron 1, was ≈3.6 kb in length. The stop codon was identified at position 2374–2376 and the polyA tail addition site at position 2454–2460 (Fig. 1). Comparison of the cDNA and genomic sequences allowed identification of the intron/exon splice sites (Fig. 1). Seven exons were identified. They are designated as exon 1, 2, 3, 4a, 4b, 5, and 6 and are 79, 240, 655, 132, 187, 132, and 720 bp in length, respectively. Six introns were identified, which is consistent with the structure of other tephritid *white eye* genes (Xiao 1997, Bennett and Frommer 1997). The five introns sequenced were designated as intron 2, 3, 4a, 4b, and 5 and were 71, 84, 55, 68, and 58 bp in length, respectively. Labels for *white eye* introns and exons are derived from the *D. melanogaster* model (Bennett and Frommer 1997).

Mutant *white eye* Allele Consensus Sequence. Comparison of the wild-type and mutant allele cDNA sequences indicated a difference in the coding region of the gene. The cDNA sequence from the mutant allele included intron 4b. The sequence from wild-type and mutant allele RT-PCR products in this region were compared with PCR products from the genomic DNA. The splice site, at intron 4b, contained a single base pair substitution in the mutant allele. The "T" at position 1505 in the wild-type was an "A" in the mutant (Fig. 1). Correct splicing of the introns during transcription of RNA is dependent on a conserved sequence: "GT. . . . AG" (Mount 1982, Keller and Noon 1985). In the mutant allele of the *white eye* gene of *B. cucurbitae*, the 5′ "GT" splice site was not present; thus, intron 4b was not excised from the RNA.

Northern hybridization data (Yang, S.D.M., and Saul, unpublished data) suggested the presence of intron 4b in the mRNA. The transcript from the mutant allele of *B. cucurbitae* was slightly larger than that of the wild type. This difference in transcript size can be accounted for by the presence of intron 4b in the mRNA of the mutant allele. It also showed that the mutant allele produced a transcript with levels of expression similar to the wild-type allele.

Comparison of PCR products from the cDNA and genomic DNA were used to confirm the presence of intron 4b in the mutant transcript. RT-PCR and PCR products were amplified from the mutant and wild-type sequences by using the same primer pairs that spanned the regions containing intron 4b and intron 5. These PCR products were separated on a 3% agarose gel (Fig. 2). PCR products from the genomic DNA of both alleles were approximately the length of the sequenced lengths of 954 bp (Fig. 1). The RT-PCR product from the wild-type allele had an approximate length consistent with the sequenced intron/exon lengths of 828 bp. However, the RT-PCR product from the mutant allele had an approximate length of 900 bp. This estimated difference is similar to the sequenced length of intron 4b (68 bp; Fig. 1). This is compelling evidence that the *white eye* mutant phenotype of *B. cucurbitae* results from the presence of intron 4b in the mRNA.

Promoter Region. Sequence upstream of the start codon in the wild-type *white eye* allele of *B. cucurbitae* was examined for possible initiation and promoter regions based on homology to sites in the *D. melanogaster white* gene. The *D. melanogaster white* gene does not contain a TATA-box, but it does have a 6-bp consensus initiator (Inr) sequence: "TCA(G or T)T(T or C)" (Ohtsuki et al. 1998). No TATA-box was identified in the upstream sequences of the *B. cucurbitae white eye* allele. However, an Inr sequence identical to that of *D. melanogaster* was identified. The sequence, "TCAGTT," is located at −1098 to −1093 (Fig. 1). The placement at this position is consistent with initiation of the transcript to produce a 3.6-kb sequence. The Inr

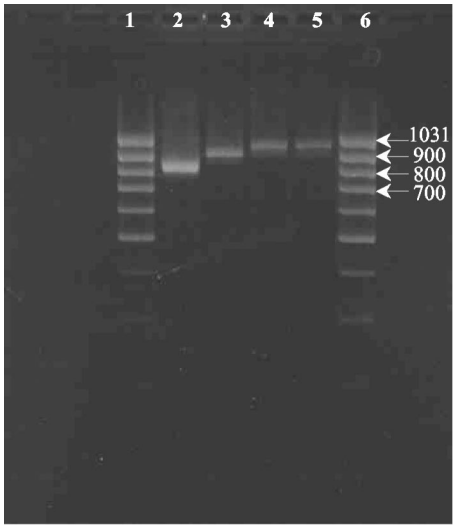

Fig. 2. Agarose gel of PCR and RT-PCR products by using identical primer sets (1319–1339 and 2253–2273) from *B. cucurbitae* wild-type and mutant alleles. Lanes 1 and 6 are 100-bp ladders (MBI Fermentas). Ladder sizes for the bands of interest are indicated on the right. Lane 2 is the wild-type RT-PCR product, sequenced at 828 bp in length. Lane 3 is the mutant RT-PCR product, sequenced at 896 bp. Lanes 4 and 5 are wild-type and mutant allele genomic PCR products, respectively, sequenced at 954 bp. Samples were run on a 3% agarose gel.

sequence identified in the *D. melanogaster white* gene and the *B. cucurbitae white eye* gene is conserved across a wide range of arthropods (Cherbas and Cherbas 1993).

Downstream promoter elements (DPEs) have been found in several TATA-less *D. melanogaster* genes (Kutach and Kadonaga 2000). The DPE sequences are believed to have strict spacing requirements, being located within +28 to +33 bp relative to the Inr sequence in *D. melanogaster.* The consensus of the DPE sequence is "G(A or T)CG." The sequence "GTCG" was located at +40 to +43 bp relative to the Inr sequence (Fig. 1) in the *B. cucurbitae white eye* gene. This is slightly outside the strict spacing determined in *D. melanogaster* but could be species specific in *B. cucurbitae* DPE sequences (Kutach and Kadonaga 2000). There are other positions located between the initiation site and the DPE that are biased toward certain nucleotides in *Drosophila* DPE-containing promoters. These include a "T" at +17, a "G" at +19, and a "G" at +24. These nucleotides were found at their respective positions in the *B. cucurbitae white eye* gene as well. In summary, the *B. cucurbitae white eye* promoter region is TATA-less, contains an Inr, and has a potential DPE at position +39 to +42 bp relative to the Inr.

5′ Untranslated Region (UTR) Conserved Sequences. The long 5′ UTR of the melon fly *white eye* transcript is one-half the length of the coding region and may play an important regulatory role in the expression of the *white eye* gene. Five regions seem to be conserved in both sequence composition and relative distance upstream of the start codon compared with *B. tryoni* and *C. capitata* (note that *D. melanogaster* has a very short 5′ UTR). The first region, from −508 to −425 (Fig. 3), has a 96% identity in *B. tryoni.* The second region, from −396 to −331, has an identity of 99% in *B. tryoni* and 91% in *C. capitata.* The third region, from −309 to −246, has an identity of 98% in *B. tryoni* and 86% in *C. capitata.* The fourth region, from −210 to −167, has an identity of 86% in *B. tryoni* and 77% in *C. capitata.* The fifth region is from −83 to −33 and has an identity of 86% in *B. tryoni* and 65% in *C. capitata.* What is significant in these similarities is that they are all noncoding regions that have been conserved in both sequence and relative position in the untranslated leader section. In comparison with *B. tryon* and *C. capitata*, these five regions are the only similarity in exon 1 upstream of the start codon. The remaining sequences further upstream to the *Inr* sequence, and the sequence in between these five regions, show no similarity. Because the organisms have kept these long 5′ UTRs in their transcripts with these conserved regions, they may be part of the complex regulatory control needed for the tissue- and temporal-specific expression of the *white eye* gene. Alignments were made using MultiAlin webtool (Corpet 1988).

Putative *white eye* Protein. The *B. cucurbitae* wild-type consensus sequence was used to predict the putative protein sequence. The putative protein is 679 amino acids in length and contains distinct regions characteristic of membrane transport proteins, the ATP-binding cassette, and transmembrane regions. The ATP-binding cassette contains the Walker A, Walker B, and ABC motifs, which are responsible for binding and processing ATP (Higgins 1992). The processing of ATP is thought to cause the protein to undergo a conformational change (Hyde et al. 1990). This change in conformation effectively opens the transmembrane channel that is formed by six hydrophobic transmembrane regions and allows transport of ommochrome or pteridine precursors.

A comparison of the *B. cucurbitae* putative protein and those of four other Diptera, *B. tryoni*, *C. capitata*, *D. melanogaster*, and *Lucilia cuprina* (Wiedemann) was conducted with BLAST (GenBank). The tephritids (*B. tryoni*, *B. dorsalis*, and *C. capitata*) had a high degree of homology (94–97%) to the *B. cucurbitae white eye* gene. *D. melanogaster* and *L. cuprina* have 82 and 80% homology to the *B. cucurbitae white eye* gene, respectively. Significantly, these two proteins had longer amino acid chains (697 and 699) than those from the tephritids, which accounted for much of the difference. These results are consistent with phylogenic relationships; because the more closely related organisms have a higher degree of homology.

The transmembrane regions of the putative *white eye* proteins are highly conserved across eukaryotes (Higgins 1992). The six hydrophobic regions that would be located inside plasma membrane are at nucleotide positions 1494–1616, 1655–1771, 1843–1903, 1936–1993, 2023–2080, and 2293–2350. The six trans-

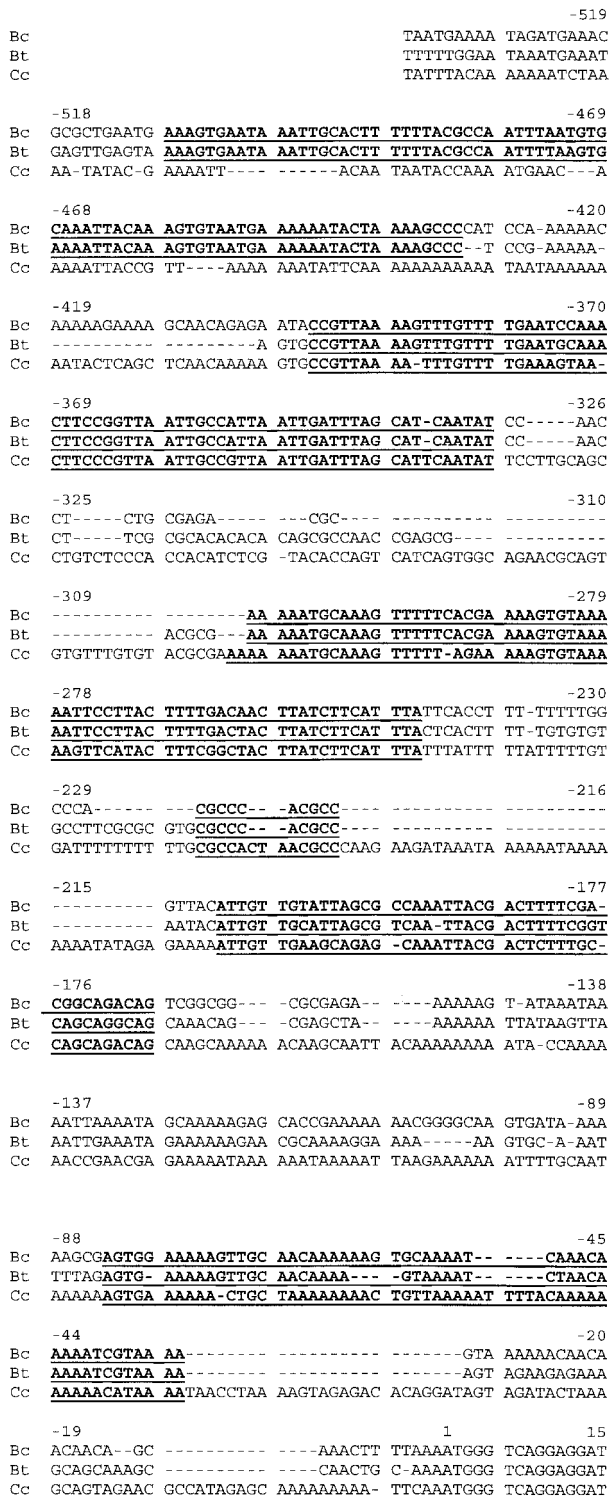

Fig. 3. Multiple sequence alignment of exon 1 untranslated leader regions from *B. cucurbitae* (Bc), *B. tryoni* (Bt), and *C. capitata* (Cc). Base pair positions are numbered relative to the start codon, ATG, in *B. cucurbitae*. Conserved sequences from Bc, Bt (GenBank accession no. U97104) and Cc (GenBank accession no. AF318275) are highlighted in bold and underlined.

membrane regions are similar among the dipteran species compared. Similar regions also have been identified in the *scarlet* and *brown* proteins (Ewart et al. 1994).

In conclusion, the goals of this research were to obtain the sequence of the melon fly mutant and wild-type *white eye* alleles and to identify the molecular basis of the mutant *white eye* allele. The sequence of the mutant and wild-type *white eye* alleles of *B. cucurbitae* was obtained and a comparison of the coding regions was made. Seven exons (1, 2, 3, 4a, 4b, 5, and 6) and six introns (1, 2, 3, 4a, 4b, and 5) were identified, similar to other dipteran *white eye* genes. The coding region of the wild-type *white eye* allele was 2.1 kb in length and encodes a putative protein of 679 amino acids.

The mutant *white eye* allele was similar in expression, length, and structure to the wild-type transcript. However, the mutant allele had a single base pair mutation at position 1505. The altered base pair disrupts the RNA splice recognition site so that intron 4b is not removed from the RNA transcript. Translation of such an mRNA would result in a truncated putative protein because the intron sequence introduces a premature stop codon, terminating protein synthesis at amino acid 448. Transport of pigments from both the ommochrome and pteridine pathways would be blocked, resulting in a *white eye* phenotype.

We believe that the RNA splice site interruption is the basis for the *white eye* mutation. Initial isolation and backcrossing of mutant strain suggested that the mutation was based on a single recessive gene. Our studies have shown that the expression of the mutant and wild-type mRNA transcripts is similar but that the mutant transcript contains additional RNA. However, the genomic composition and structure of the *white eye* alleles are similar, indicating no insertions or deletions in the mutant sequence. In addition, the original mutation was isolated from a wild-type line, without radiation or chemical treatments for inducing mutations. Thus, it is unlikely that two mutations spontaneously occurred in the same strain. This suggests that the *white eye* phenotype we have observed in the melon fly is a result of a single base pair mutation.

Further research to confirm this finding would include mutant rescue with germline transformation by using a transposable element. Successful mutant rescue would validate this strain as a potential marker for transformation. Additional studies also are warranted in comparing the untranslated leader section of the melon fly *white eye* gene with other tephritids and other transmembrane proteins, such as the *scarlet* and *brown* genes. Although we were able to find similarities in the *white* eye genes of *B. tryoni* and *C. capitata*, we did not find any similar regions in the untranslated sections of *scarlet* genes deposited in GenBank. The conserved regions found in this study may play an important role in the temporal and physiological expression of the *white eye* gene.

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